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Biomedical Engineering

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Complementary DNA assay

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Table of contents

Complementary DNA Assay

1 Considerations & Assumptions

- For the click reaction with the DBCO-modified DNA, the same conditions (same concentrations and ratio to the number of cells) as for the click reaction with DBCO-PEG_4-TAMRA. Therefore, the concentration of DBCO-modified DNA should be 30 µM total and the concentration of cells should be $1*10^9$ cells per ml during the click reaction.
- During plate reader measurement, it is preferred to have approximately 1 nmol of protein in each of the wells. Rough estimates lead to the assumption that approximately $3*10^5$ cells per well are needed to obtain this amount of proteins:

Figure	Value	Unit
Proteins in every well	$1.00*10^{-9}$	M
Volume in every well	$5.00*10^{-5}$	l
[Proteins] in every well	$5.00*10^{-14}$	mole protein
Constant of Avogadro	$6.02*10^{23}$	per mole
Number of proteins per well	$3.01*10^{10}$	
Number of proteins per cell	$1.00*10^5$	
Number of bacteria in each well	$3.01*10^5$	cells
Stock concentration of clicked	$3.01*10^7$	cells per ml

2 Preparation of cells

Estimated bench time: 45 minutes
Estimated total time: 2 hours
Purpose: Clicking and diluting the cells with DBCO-modified oligonucleotides.

2.1 Materials

- Bucket with ice
- Cell Density Meter (OD600)
- Cuvette
- ddH_2O
- Eppendorf tubes
- MiniSpin Centrifigue
- Multiwell pipette
- pAzF-functionalized cells
- PBS-BSA (0.5%)
- Pipettes and tips
- Tabletop Centrifuge

2.2 Setup & Protocol

- Spin down the cells in the tabletop centrifuge for 15 minutes at 3000 xg and 4 °C.
- Discard the supernatant.
- Resuspend the cells with 1 ml 0.5% PBS-BSA.
- Transfer the mixture to an Eppendorf tube.
- Spin for 1 minute at 13,400 rpm.
- Discard the supernatant
- Resuspend the cells with 200 ul of 0.5% PBS-BSA.
- Perform an OD600 measurement on a 20X dilution of the culture sample. The OD600 measurement should be done with:
  - Blank: 950 ul ddH_2O and 50 ul 0.5% PBS-BSA
  - Sample: 950 ul ddH_2O and 50 ul 0.5% PBS-BSA
- Multiply the OD600 with 20.
- Calculate the amount of cells in the culture by using the Agilent Technologies website.
- Make a dilution with a concentration of $1.43*10^9$ cells/ml.

3 Preparing stock solutions for furimazine & oligos

Estimated bench time: 25 minutes
Estimated total time: 25 minutes
Purpose: Make dilutions for the DBCO-modified DNA strands, furimazine and the oligos complementary to both DBCO-modified DNA strands.

3.1 Materials

- DBCO-modified DNA strands (diluted to 100 M)
- Eppendorf tubes
- Furimazine
- PBS-BSA (0.5%)
- Pipettes and tips

3.2 Setup & Protocol

- Make a DBCO-DNA stock solution where both oligos are mixed in a 1:1 ratio. Vortex the mixture such that the DBCO-DNA stock solution is well mixed.
- Make a furamizine stock by diluting 1 volume unit of furimazine with 9 volume units of 0.5% PBS-BSA.
- Make 30 µl of a 10240 nM (=2048 nM * 5) dilution of the desired oligo.

4 Click reaction

Estimated bench time: 10 minutes
Estimated total time: 10 minutes
Purpose: To click the DBCO-modified DNA to the pAzF-functionalized cells.

4.1 Materials

- 1:1 dilution of the DBCO-modified DNA
- Bacteria solution
- Eppendorf tubes
- Heat/shaking-block
- Pipettes and tips

4.2 Setup & Protocol

- Prepare the following mixtures in an Eppendorf tube. *The first tube serves as a negative control. The second tube will contain cells with clicked oligos. More cells are clicked than necessary for the assay. This is done such that not only the ratio between DBCO-modified DNA and cells remains the same as in the FACS experiments, but also the concentrations.*

Tube	PBS-BSA (0.5%)	DBCO-DNA (100uM stock)	Bacteria (1.43*10^9 cells/ml)
1	30 µl	0	70 µl
2	0	30 µl	70 µl

- Incubate both tubes at 4 °C for one hour at 300 rpm.

5 Preparing the wells plate

Estimated bench time: 1 hour
Estimated total time: 2 hours
Purpose: To make the correct dilutions in the wells plate, this will be used for the measurement.

5.1 Materials

- Clicked cells
- Furimazine diluted in PBS
- Multipipette
- Negative control (unclicked cells)
- PBS-BSA (0.5%)
- PerkinElmer 384 Flat Bottom White wells plate
- Pipettes and tips

5.2 Setup & Protocol

- The following table shows the layout of the wells plate as it will be prepared.

Concentratie in nM	0	1	2	4	8	16	32	64	128	256	512	1024	2048
Oligo 5 (=Spacer 6bp) NB													
Oligo 5 (=Spacer 6bp) NB													
Neg. Control NB (Oligo 5)													
Oligo 4 (=Spacer 3bp) NGNL													
Oligo 6 (=Spacer 9bp) NGNL													
Neg. Control NGNL (Oligo 4)													

- Furimazine, 0.5% PBS-BSA and the cells will be inserted into the plates with the multipipette. The multipipette is orientated horizontally and will be used from top to bottom. To pipette up with the multipipette, prepare the following in another wells plate.

Materiaal	0	1	2	4	8	16	32	64	128	256	512	1024	2048
Clicked NanoBiT	50	50	50	50	50	50	50	50	50	50	50	50	50
NanoBiT without click	20	20	20	20	20	20	20	20	20	20	20	20	20
Clicked NGNL	50	50	50	50	50	50	50	50	50	50	50	50	50
NGNL without click	20	20	20	20	20	20	20	20	20	20	20	20	20
Furamizine PBS	80	80	80	80	80	80	80	80	80	80	80	80	80

- Dilute the clicked cells to a concentration of $3*10^7$ cells/ml by combining 30 µl of clicked bacteria with 970 µl of 0.5% PBS-BSA.
- Fill the wells plates in the following manner.
    - Add 10 µl of 0.5% PBS-BSA to all the wells except for the leftmost and rightmost wells.
    - Add 20 µl of the correct oligo (10.24 µM) to the rightmost well.
    - Take a 10 µl pipette and pipette slowly up and down in the rightmost well. Make sure that the pipette is filled with 10 µl again and move one well to the left. Pipette slowly up and down again and transfer 10 µl to the next well. Repeat until all wells except for the leftmost well are filled with 10 µl oligo.
    - Take the multipipette, pipette 15 µl of the correct ells from plate 2 and pipette 10 µl of cells down in the corresponding wells in well plate 1. Discard the multipipette points. Repeat until all the wells are filled with 10 µl of cells.
    - Take the multipipette, pipette 25 µl of PBS up from a container and pipette 20 µl of PBS down in all rows in plate 1. Make sure to discard the pipette points after each row. Repeat until all wells contain PBS-BSA.
    - Add 10 µl of PBS to the leftmost wells with a normal pipette.
    - Incubate the cells in the cold room for an hour. Shake the cells at 4 °C such that they do not sink to the bottom of the well.
    - Using the multipipette, add 10 µl of the diluted furimazine to each of the wells.

6 Measurement

Estimated bench time: 5 minutes
Estimated total time: 1.5 hours
Purpose: Measurement of the luminescence and fluorescence of the samples in the Tecan Infinite F500 plate reader.

6.1 Materials

- Plate prepared using the above protocol
- Tecan infinite F500 plate-reader

6.2 Setup & Protocol

- Insert the plate into the plate reader.
- Make sure to select the correct plate from the interface of the Tecan infinite F500 plate-reader.
- Select the wells which contain the samples.
- Select dual luminescence measurement with the filters Green and Magenta (green is used to measure mNeonGreen activity, Magenta is used to measure NanoLuc's activity).
- End measurement with orbital shaking for 5 seconds with an amplitude of 2.5 mm to ensure that the cells do not sink to the bottom of the wells.
- Repeat the measurement for 30 cycles to obtain enough results.